Correcting a mistake: *Limnophyes stagnum* Namayandeh, Guerra & Ram, 2024 is not conspecific with *Limnophyes* sp. 14ES

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Abstract

In this communication, we report on correcting DNA barcoding records of *Limnophyes stagnum* Namayandeh, Guerra & Ram, 2024. The five sequences we submitted to BOLD and published in Namayandeh et al. (2024) under *L. stagnum* match the CO1 sequences of *Limnophyes* sp. 14ES and the two species are not conspecific. Both species were collected from the type locality of *L. stagnum* but at different times with a misassumption that both species represent a single taxon. We have corrected our records in BOLD, replacing the names of the five sequences with *Limnophyes* sp. 14ES. Additionally, we obtained and uploaded a single CO1 sequence of *L. stagnum* in BOLD and incorporated it into new molecular analyses reported here. Based on our results, the closest sequences to *L. stagnum* were those of *Limnophyes natalensis* (Kieffer, 1914). The minimum K2P distance of *L. stagnum* with *L. natalensis* was 10.0% (average 11.4%), large enough to support the delimitation of *L. stagnum* sp. nov. from *L. natalensis*. Further discussion on the morphological differences of the two species and those of *L. stagnum* and *Limnophyes* sp. 14ES are provided.

Results and Discussion

It was brought to our attention by Dr. Elisabeth Stur of NTNU that the five sequences we submitted to BOLD and published in Namayandeh et al. (2024) as sequences of *Limnophyes stagnum* Namayandeh, Guerra & Ram, 2024 match the CO1 sequences of *Limnophyes* sp. 14ES. Dr. Stur communicated to us that based on her examination, the females of *Limnophyes* sp. 14ES are morphologically different from *L. stagnum*. It is thus likely that two species of *Limnophyes* were present in Detroit's Palmer Park vernal pool when we investigated it (Namayandeh et al. 2024). We assumed that the sequences of *Limnophyes* sp. 14ES females belonged to *L. stagnum* without examining the morphology of the adult *Limnophyes* females of the specimens which were sequenced and uploaded in BOLD under the name of our new described species *L. stagnum*. Both species were collected from the type locality of *L. stagnum*, but at different times. *Limnophyes* sp. 14ES was sampled in the spring, and *L. stagnum* in the fall. We have corrected our records in BOLD and replaced the names of our sequences with *Limnophyes* sp. 14ES. In addition, we uploaded a single CO1 sequence of *L. stagnum* that we incorporated into a new molecular analysis (Fig.1).

Details of the methodology used in the molecular analysis are given in Namayandeh et al. (2024). The CO1 sequence of the *L. stagnum* specimen on which we confirmed its unique morphology has the sample ID PPA19 and the process ID DTPPA019-25 in BOLD.

The two species' most apparent differences are their coloration and chaetotaxy. *L. stagnum* is uniformly dark brown while *Limnophyes* sp. 14ES is much lighter and greyish brown. The adult females of *Limnophyes* sp. 14ES have diagonally placed preepisternals below the epimeral region, whereas the ones of *L. stagnum* have horizontally placed preepisternals close to the anapleural suture (Fig. 2A-B). Moreover, the females of *Limnophyes* sp. 14ES have a five-segmented antenna, whereas the ones of *L. stagnum* have a four-segmented antenna (Fig. 2C). Other corrections are the geographical records of *L. stagnum. Limnophyes stagnum,* for now, is only reported from the type locality whereas *Limnophyes* sp. 14ES, seems to be widespread in the Holarctic.

The NJ analyses of *Limnophyes* sequences from Pond A (i.e., *L. stagnum* and *L.* sp. 14ES) and those obtained from NCBI and BOLD produced the same tree topology (Fig. 1). The six sequences of *L.* sp. 14ES, from Pond A, clustered with three sequences identified as *Limnophyes* sp. process ID CNTIC4604-15, JSJUN346-11, and NCCA2089-11 from Ontario, Canada (Hebert et al. 2016, deWaard et al. 2019). The

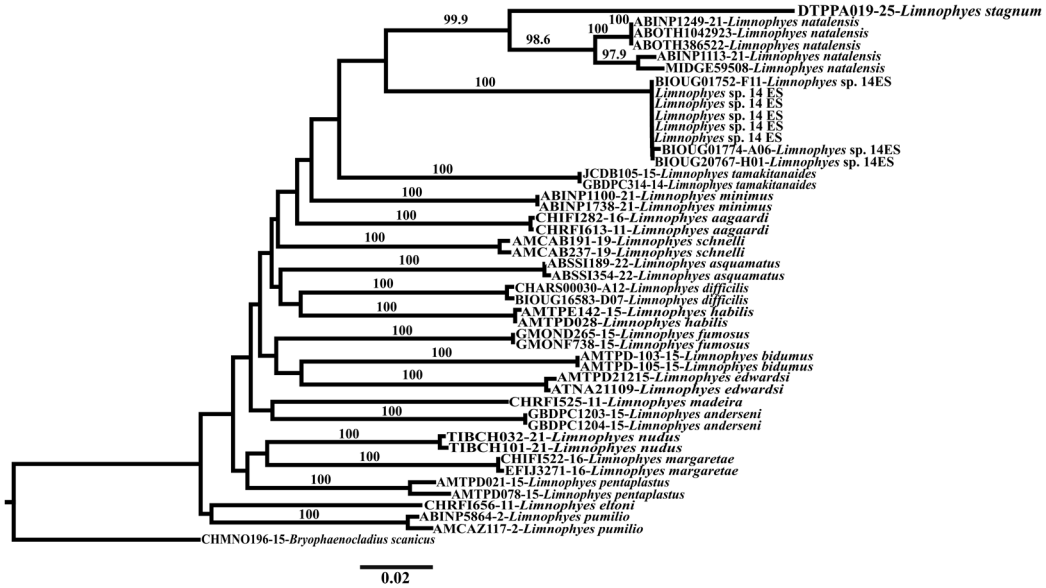

Figure 1. Neighbor-Joining (NJ) tree of *Limnophyes* Eaton species and one outgroup *Bryophaenocladius scanicus* (Brundin, 1947) inferred from the COI DNA barcode marker (658 bp). Numbers on branches represent the bootstrap value for Neighbor-Joining (NJ) using 10000 replicates; numbers < 95 were omitted.

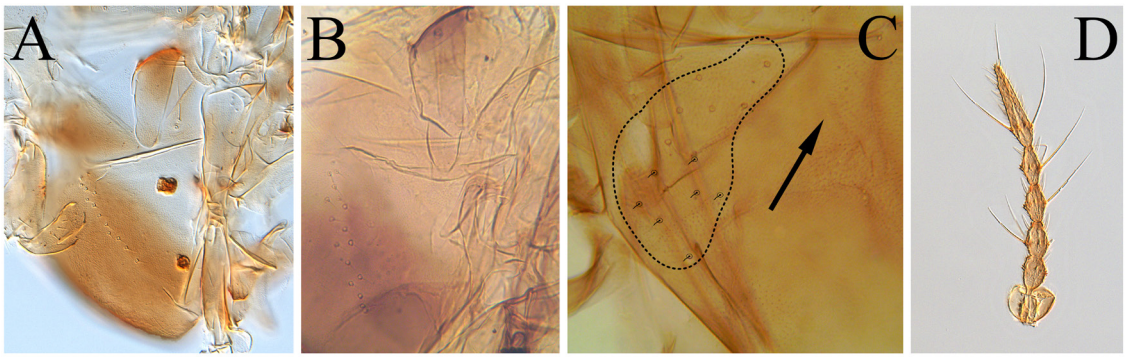

Figure 2. Comparison of some relevant female characteristics of *Limnophyes* Eaton. *Limnophyes* sp. 14ES (A, B, D); *Limnophyes stagnum* Namayandeh, Guerra & Ram, 2024 (C). A-C. Thorax preepisternals (arrow indicates the orientation of the thorax). D. Antenna. A & D specimens are from Norway; B & C specimens are from Michigan, USA. Images A & D are courtesy of E. Stur.

single sequence of *L. stagnum*, from Pond A did not cluster closely with any known sequence from BOLD or NCBI. The closest sequences to *L. stagnum* were those of *Limnophyes natalensis* (Kieffer, 1914), BOLD process id's ABINP1113-21, MIDGE595-08, ABOTH3865-22, ABINP1249-21, and ABOTH10429-23. The maximum intraspecific K2P pairwise distances calculated for the five sequences of *L. natalensis* was 1.7%. The maximum intraspecific K2P pairwise distances calculated for the six sequences of *L.* sp. 14ES, and the two sequences of *Limnophyes* sp. from Ontario were 0.05%. The minimum K2P distance of *L. stagnum*, with *L. natalensis* was 10.0% (average 11.4%). The overall mean distance of all *Limnophyes* species was 15.0%

The minimum and average interspecific distances of *L. stagnum* with *L. natalensis* are much lower than the overall mean distance of all *Limnophyes* analyzed. This may suggest that molecular methods may not clearly separate the two species. However, the minimum distance of 10.0% obtained based on the distance-based methods of K2P is large enough to support the delimitation of *L. stagnum* sp. nov. from *L. natalensis*, and it is consistent with the interspecies barcode gap in Chironomidae (Ekrem et al. 2010; Montagna et al. 2016). Additionally, the lower interspecific distances of *L. stagnum* with *L. natalensis* could stem from the fact that we only had one sequence of *L. stagnum* in our analyses, and with more sequences, this distance could vary. There is also a possibility that the two species have diverged from one another quite recently, and this has caused a low genetic divergence (Lin et al. 2015). The morphology supports that *L. stagnum*

and *L. natalensis* are two different species. The adult males of *L. stagnum* can be separated from that of *L. natalensis* by a combination of the following characteristics: Antenna 10 segmented, AR 0.8; costa extension 62 µm long; lanceolate setae absent, humerals absent, gonostylus expanded evenly from base to apex, crista dorsalis very narrow. *L. natalensis* adult males have a 12-segmented antenna with AR of 0.3–0.5; costa extension 25–53 µm long; lanceolate setae present; humerals 6; gonostylus not expanded, crista dorsalis pronounced and pointed.

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